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Re-investigation of the fragmentation of protonated carotenoids by ESI and nanoESI-CID-MS/MS

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Abstract

RATIONALE Carotenoids are polyene isoprenoids with an important role in photosynthesis and photoprotection. Their characterization in biological matrices is a crucial subject for biochemical research. In this work we report the full fragmentation of 16 polyenes (carotenes and xanthophylls) by electrospray ionization tandem mass spectrometry (ESI-CID-MS/MS) and nanospray tandem mass spectrometry (nanoESI-CID-MS/MS).

METHODS Analyses were carried out on a quadrupole time-of-flight (QToF) mass spectrometer coupled with nanoESI source and on a Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer with ESI source. The formulae of the product ions were determined by accurate-mass measurements.

RESULTS It is demonstrated that the fragmentation routes observed for the protonated carotenoids derive essentially from charge remote fragmentations and pericyclic rearrangements, such as electrocyclic and retro-ene eliminations (assisted or not by a sigmatropic hydrogen shift). All mechanisms are dependent of *cis-trans* isomerization through the formation of several conjugated polyene carbocation intermediates. Some specific ions for the carotenoid epoxides were justified through formation of cyclic oxonium ions.

CONCLUSION Complete fragmentation pathways of protonated carotenoids by ESI and nanoESI-CID-MS/MS provide structural information about functional groups, polyene chain and double bonds, and contributes to carotenoid identification based on MS/MS fragmentation patterns.

Keywords: carotenoids, retro-ene, aromatic elimination, tandem mass spectrometry, electrospray ionization.

Introduction

Carotenes and xanthophylls (oxygenated carotenoids) represent a large group of 40-carbon isoprene natural products with conjugated polyene chain (with up to 15 double bonds).[1] These molecules are widely distributed in nature where they play important roles in photosynthesis and photoprotection as direct quenchers of singlet oxygen and other reactive oxygen species (ROS), vitamin A precursors, membrane stabilizers, light harvesting and thermal dissipative agents.[2-7]

About 750 naturally occurring carotenoids had been reported so far, with biosynthetic sources ranging from bacteria, yeast, algae and plants. In animals and humans their accumulation is derived through dietary intake. Over 250 carotenoids have been isolated from marine organisms, some of them (e.g. acetylenic carotenoids) come exclusively from this source.[6, 8]

According to literature, most structural variations on carotenoids arise from biosynthetic modifications at the polyene chain, like cyclization, hydroxylation, epoxidation and rearrangement, which led to differences in the carotenoid backbone, the presence of heteroatoms and/or changes in π-system size and position.[1, 9, 10]

Considering their economic relevance to the pharmaceutical and cosmetic industries, numerous analytical methods have been developed to detect and quantify carotenoids based on their chemical reactivity and light-absorbing properties.[11] High performance liquid chromatography coupled with photodiode array detector (HPLC-PDA) is by far the most common method, nevertheless structural similarity and isomeric forms hinder identification, especially in mixtures, where complete LC resolution is mandatory to assure correct detection solely by UV-Vis spectra.[11, 12]

Liquid chromatography combined with mass spectrometry (LC-MS) is a step forward for carotenoid characterization since it provides unique structural features in an selective and

sensitive analysis, promoting the distinction between co-eluting carotenoids in small amounts of very low concentration samples.[11, 13]

Various MS methods have been reported for carotenoid analysis using different ionization methods, including electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), electrospray (ESI) and atmospheric pressure chemical ionization (APCI).[13-20] Most of these applications are focused on molecular weight determination and/or rapid bulk analyses to confirm their presence in complex matrices. Only a few recent papers have looked into the use of MS/MS to differentiate structural isomers and identify new compounds.[13, 17, 18, 20] Moreover, very few studies have explored mechanistically and structurally the gas-phase chemistry of carotenoids to understand their unique fragmentation patterns.[13, 17, 21]

One possible reason might be the ability of carotenoids to oxidize through electron abstraction, producing $M^{\bullet+}$, as well as generating protonated ions through acid-base ionization. This opens up multiple fragmentation routes resulting from these distinct ionization mechanisms (acid-base and/or redox) which can have the effect of hampering structural elucidation through increase complexity.[12, 21, 22]

In this study, the main fragmentation pathways of protonated carotenoids are investigated. CID-MS/MS analyses were performed on both ESI-FTICR and nanoESI-QToF instruments to produce accurate-mass data to enable the elucidation of fragmentation routes from the distinct ionization processes.

Experimental

Materials

Alloxanthin, antheroxanthin, canthaxanthin, cryptoxanthin, diadinoxanthin, diatoxanthin, lutein, neoxanthin, peridinin, prasinoxanthin, violaxanthin, zeaxanthin, ethyl-apo-carotene

and fucoxanthin were obtained from DHI Water & Environment (Copenhagen, Denmark). β-carotene and astaxanthin were purchased from Sigma-Aldrich (St Louis, MO, USA). Methanol and acetonitrile were of HPLC grade and obtained from Aldrich (Gillingham, Dorset, UK). Deionised water was used throughout the study. Stock solutions of alloxanthin (~1.4 mg mL^{-1}), antheroxanthin (~0.6 mg mL^{-1}), canthaxanthin (~0.6 mg mL^{-1}), cryptoxanthin (~1.0 mg mL^{-1}), diadinoxanthin (~1.2 mg mL^{-1}), diatoxanthin (~0.9 mg mL^{-1}), lutein (~1.3 mg mL^{-1}), neoxanthin (~1.1 mg mL^{-1}), peridinin (~1.0 mg mL^{-1}), prasinoxanthin (~1.3 mg mL^{-1}), violaxanthin (~0.8 mg mL^{-1}), zeaxanthin (~0.6 mg mL^{-1}), β-carotene (~1.3 mg mL^{-1}) astaxanthin (~1.0 mg mL^{-1}) ethyl-apo-carotene (~0.8 mg mL^{-1}) and fucoxanthin (~1.3 mg mL^{-1}) were prepared in methanol and stored at 4 °C. Prior to analysis the dilute standard was prepared in methanol-acetonitrile–water (2:1:1 v/v) with 0.5% formic acid to final concentration of approximately 0.5 mg mL^{-1}.

Instrumentation

Nanospray ionization analysis was performed on a QStar-XL quadrupole time-of-flight hybrid instrument (Applied Biosystems, Warrington, UK) using a Nanomate HD automatic chip based nanospray system (Advion Biosciences, Norwich, UK). The QStar acquisition parameters were: ion source gas flow rate, 50 arbitrary units; curtain gas flow rate, 20 arbitrary units; ionspray voltage, 2700 V; declustering potential, 75 V (except where indicated otherwise); focusing potential, 280 V; and declustering potential 2.15 V. The ion source gas and curtain gas were nitrogen.

High-resolution accurate-mass ESI analyses were performed on a 7 Tesla Apex IV Fourier transform ion cyclotron resonance instrument (Bruker Daltonics, Billerica, MA, USA) using an Apollo off-axis ESI source. Samples were directly infused from a syringe pump at 100 µL min^{-1}. The acquisition parameters were: capillary voltage, 4600 V; end plate voltage,

3500 V; capillary exit potential, 200 V and drying gas temperature, 200 °C. Spectra were obtained by summing 80 0.5 s scans.

In all cases, CID-MS/MS fragmentation analyses were performed on the isolated parent ions using either N_2 or CO_2 collision gas.

Results and Discussion

Carotenoids can produce both molecular ions, $M^{\bullet+}$, and protonated molecules, $[M+H]^+$, in positive mode ESI independent of the capillary voltage.[12, 19, 21-23] This ability to be either oxidized (through abstraction of one electron) or to be protonated (in acid-base ionization) is significantly influenced by charge delocalization by the conjugated polyene chain and the oxygen atoms.[12, 19, 21-23] Depending on the type of ions generated (protonated or radical), CID-MS/MS can generate multiple distinguishable fragmentation routes based on gas-phase acid-base and/or redox chemistry.[12, 17] In order to avoid $M^{\bullet+}$ formation and the subsequent redox reactions in MS/MS experiments, it has been suggested that acidified protic solvents are used to aid protonation and to eliminate the generation of the radical molecular ion.[12, 22] In this work, all the carotenoids standards (see figure 1) were dissolved in a mixture of protic solvents acidified with 0.5% formic acid prior to ionization. The results clearly showed that the use of protic solvents favorthe production of $[M+H]^+$ in both ESI and nanospray ionization.[12, 19, 22]

The $[M+H]^+$ ions were selected as the precursor ions for ultra-high resolution FTICR-MS/MS. The use of high resolution mass spectrometers enables the full separation and isolation of $[M+H]^+$ from any radical ions that are still generated, and so avoiding the occurrence of cross radical fragmentations that can result from low resolution precursor ion selection.[17] MS/MS analyses were also conducted by nanoESI-CID-QToF. The major product ions from tandem mass spectra of all carotenoids (available in Supporting

information) are summarized in Table 1, and the major fragmentation routes are exemplified in scheme 1.

Various product ions are present in the MS/MS analysis of all the carotenoids, specifically in the low-mass region (*m/z* < 300). Similar product ions were obtained in both ESI and nanoESI, despite nanoESI producing more abundant low-mass ions. Previous studies have compiled positive ion fragmentation data of carotenoids using various ionization techniques and matrices, however no systematic studies have completely elucidated their gas-phase chemistry.[13, 17]

The suggested fragmentation routes are initiated by the elimination of molecules of water from the oxygenated groups (hydroxyl, carbonyl or oxirane substituents) in xanthophylls. Dehydrations from hydroxyl groups can be explained via 1,4-elimination, except for lutein, where the hydroxyl group is in a allylic position which would promote direct protonation and formation of an allylic carbocation (stabilized through resonance) after water elimination.[21, 24, 25] Neutral eliminations of acetic acid or CO can also be observed for peridinin, fucoxanthin and astaxanthin by a similar mechanism.

Fragmentation pathways of all carotenoids were also accompanied by pericyclic reactions as electrocyclic, retro-ene eliminations and charge remote fragmentations (assisted by a sigmatropic hydrogen shift in some cases), through the formation of conjugated carbocation ions with different *cis-trans* isomerization of the polyene chain.[26, 27]

For pericyclic reactions to occur, several pairs of bound electrons need to be able to move concertedly in a six-membered transition state; which requires adequate orientation of the orbitals involved.[28, 29] Additionally, for retro-ene fragmentation reactions to occur, a γ-hydrogen to an unsaturated carbon is requiredto yield both -ene and -enophile fragments.[28] Both requirements (correct orbital orientation and γ-hydrogen distribution) can be achieved during the protonation of carotenoids through carbocation formation and *cis-trans* π-system conversion , as displayed in scheme 2.

Formation of the intermediate carbocation can be rationalized by protonation at a sp^2 carbon double bond, which results in sp^3 hybridization and the formation of a single bond. Subsequently, free rotation of the new single bond can reverse isomerization due to the resonance effect of the polyene chain.[27] In case of xanthophylls, isomerization can be assisted by the lone pair of electrons from the oxygen atoms.

Formally, positive charge delocalization should be drawn at a terminal double bond of one ionone moiety, e.g. C5-C6, and protonation could be equally possible at multiple sites, however semi-empirical calculations showed delocalization of intermediate carbocation is negatively affected by the torsion angle between the ionone ring and the polyene chain, reducing orbital overlap and charge delocalization. On the other hand, the large number of conjugated double bonds favored carbocation resonance in the "middle" of the polyene – C13-C13', due to higher electronic density. For those reasons, protonation of carotenoids is favored at C7-C11, even in xanthophylls, where it would be expected to observe protonation at electronegative oxygen. [26, 27]

Electrocyclic elimination was proposed as the mechanism for the neutral losses of 92 and 106 u, (elimination of toluene and xylene respectively), observed for all of the carotenoids analyzed.

The mechanism for the elimination of aromatic rings from carotenoids has been proposed as a sequential polyene 8π/6π-electron electrocyclization followed by formation of a four membered ring intermediate, as shown in scheme 3a.[21] An alternative route was recently presented, comprising of a Diels-Alder reaction forming the stable tricyclic intermediate structure as proposed in scheme 3b.[25]

Both processes require several *trans−cis* isomerizations to fulfill the correct conformation, however the symmetry-allowed disrotatory ring-closure of a cyclo-octatetraene observed in electrocylization should be energetically more favorable since it does not depend directly on the ionone ring, as proposed by [4 + 2] cycloaddition. Indeed, according to

Coughlan and co-workers (2014), density functional theory calculations at the M06-2X/cc-pVDZ level supported the electrocyclization/fragmentation cascade, probably because it avoids the steric effects of ionone ring formation. The evidence for the loss of toluene in fucoxanthin reinforces the 8π/6π electron electrocyclizations since there is no double bond in the ionone ring which is able to assist the formation of a stable tricyclic intermediate structure.[21, 24, 25]

The MS/MS analysis of all the carotenoids also displayed various abundant ions in the low-mass region (< 300 u), similar to those observed in FAB-MS/MS, EI-MS, APCI-MS/MS and ESI-MS/MS analysis.[13] These product ions have been proposed as resulting from various cleavages of the polyene chain accompanied by hydrogen transfer, however no gas-phase mechanisms have been proposed so far in the literature.[13, 18]

The rationalization for the formation of these low-mass ions suggests a mechanism pathway with several cleavages of the polyene chain by sequential retro-ene eliminations or vinyl-allyl charge remote fragmentations. The retro-ene elimination involves transfer of γ-hydrogen atom to an unsaturated center via a six-electron cyclic transition state.[28, 30] Vinyl-allyl fragmentation involves participation of an allylic hydrogen atom on a methylene group to form a six-membered transition state. The proposed mechanism of vinyl-allyl fragmentation arise from the six-membered intermediate by the transfer of the bis-allylic hydrogen atom to the alkenic carbon resulting in formation of a conjugated diene and a vinylic molecule.[28, 31-33]

Possible cleavages of the polyene chain by directed retro-ene and vinyl-allyl reaction are depicted in scheme 4. The characteristic cleavages of carbon–carbon bonds at multiple sites of the polyene chain in carotenoids are governed by protonation, extensive carbocation-charge delocalization and *cis-trans* isomerization. Beside this, hydrogen sigmatropic rearrangements are necessary to achieve proper orbital conformation and hydrogen requirements. Sigmatropic rearrangements are also pericyclic processes in

which an atom (in our case, hydrogen) σ-bonded to a carbon may rearrange its position through one or more π-electron systems, without producing any fragment ions.[28]

Carotenoid 5,6-epoxides also formed characteristic fragments at *m/z* 221 and 181. These product ions have been used as diagnostic ions for the epoxide-containing end group and its fragmentation mechanism has been described as direct 8,9 and 10,11 cleavages of the polyene chain.[13, 16-18] Here we proposed the cleavage based on epoxide-furanoxide or epoxide-oxepinoid rearrangements with formation of cyclic oxonium ions, as shown in scheme 5.

Conclusions

We have presented a complete fragmentation pathway for protonated carotenoids using ESI-CID-MS/MS. Protonation of carotenoids was produced simply through the choice of acidified protic solvents, minimizing radical molecular ion formation and undesirable redox and radical processes that may induce extra routes that complicate structural elucidation. The use of high resolution ion isolation enables the $[M+H]^+$ to be selected as the precursor ion for MS/MS studies, avoiding co-selection of any low-intensity radical ions that may still be present in the spectra.

Systematic studies of the carotenoid fragmentation pathways have demonstrated that electrocyclization elimination reactions resulted in the loss of an aromatic ring from the polyene chain. Direct cleavages of conjugated π-system also occurs by retro-ene reactions and vinyl-allyl charge remote fragmentations. All proposed mechanisms rely on correct orbital conformation and adequate distribution of hydrogens. Both requirements are achieved during protonation of the polyene chain, forming the carbocation intermediate and inducing *cis-trans* isomerization, and by sigmatropic hydrogen rearrangements.

Specific fragmentation routes included dehydratation from the ionone ring of xanthophylls and formation of oxonium ions in carotenoid 5,6-epoxides. The proposed mechanisms are based on gas-phase reactivity of carotenoids and could be of significant use for future MS/MS identification.

Supporting information

Supporting information can be found in the online version of this article.

Acknowledgments

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Figure Captions

Figure 1. Structures of carotenoids analyzed.

Scheme 1. Proposed fragmentation routes for a) astaxanthin and b) canthaxanthin. The bond colored in red is the site of the C-C cleavage.

Scheme 2. Example of a possible protonation site for the carotenoids.

Scheme 3. Carotenoid fragmentation mechanisms proposed by a) Guaratini *et al.* (2006) and b) Coughlan *et al*. (2014). In mechanism a), a sequential 8π/6π electron electrocyclization leads to an intermediate containing a four-membered ring followed by elimination of the aromatic ring, while in mechanism b), the carotenoid undergoes a [4 + 2] cycloaddition to form a tricyclic intermediate structure.

Scheme 4. Examples of a) retro-ene fragmentation in astaxanthin and b) vinyl-ally charge-remote fragmentation in canthaxanthin.

Scheme 5. Formation of oxonium ions a) *m/z* 181 and b) *m/z* 221 from carotenoid 5,6-epoxide.

1 Tables

2 Table 1. Tabulated data for the positive ion CID-MS/MS product ions from both the ESI-FTICR and nanospray-QToF analysis of the

3 protonated carotenoids. In all cases, the $[M+H]^+$ was the precursor ion.

Carotenoids	$[M+H]^+$	*Electrocyclic reaction***	*abundant ions in FTICR (first row) and QToF (second row) related to retro-ene eliminations*
1	537.4455	445, 431	261, 185, 149 321, 245, 185, 173, 135, 123
2	565.4046	473 , 459, 441	203, 215, 255, 269, 293 191, 386, 203, 363, 215, 349, 257, 307, 269, 295
3	585.4308	493, 475, 457	341, 327, 315 427, 433, 419, 175, 391, 259, 327, 315, 285, 361,351
4	553.4404	461, 447	413, 153, 187, 337, 215, 281, 269 *
5	601.4257	509, 491, 477, 473, 459	188, 429, 357 147, 187, 175, 199, 267, 413, 387, 357, 293
6	567.4197	475, 443	217, 199, 327, 315, 267, 281, 315 145, 175, 217, 199, 310, 185
7	583.4151	491	221, 413, 419, 391, 337, 327, 315 375, 199, 217
8	631.3635	-	391, 315, 210, 184 225, 329, 315, 181
9	-	477, 459	121, 159, 185, 203, 261, 315, 339, 365 165, 188, 239, 336, 282
10	569.4359	477	152, 215 175, 232, 312, 296
11	601.4257	509, 491	221, 203, 315, 419, 391

			375, 221, 203, 247, 287
12	601.4257	509, 495, 491, 477, 473, 459, 441	233, 315
			123, 167, 181, 209, 173, 221, 233, 287, 301, 349
13	597.3944	505, 491, 487, 473	413, 419, 391, 201, 285 135, 147, 201, 379, 215, 365, 285
14	659.4306	549, 531	391, 355, 275, 213, 193, 149 *
15	565.4046	473, 441	391, 327, 283, 257, 217, 199, 152, 149 *
16	461.3414	-	337, 291, 235, 221, 171, 157, 119 *

* Experiments not performed

** Ions represent elimination of toluene (92 u), xylene (106 u) and possible additional losses of water.

Figures

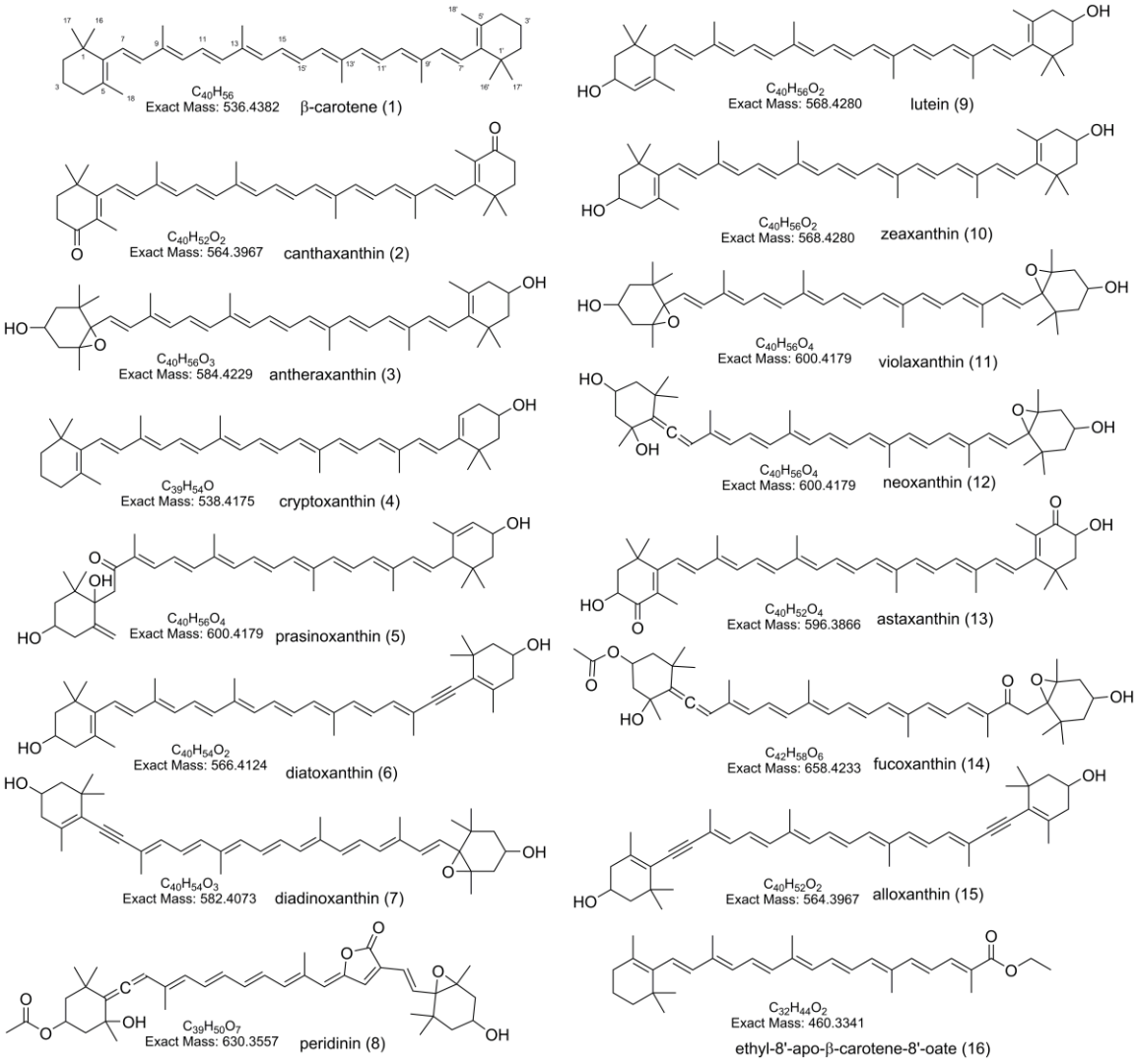
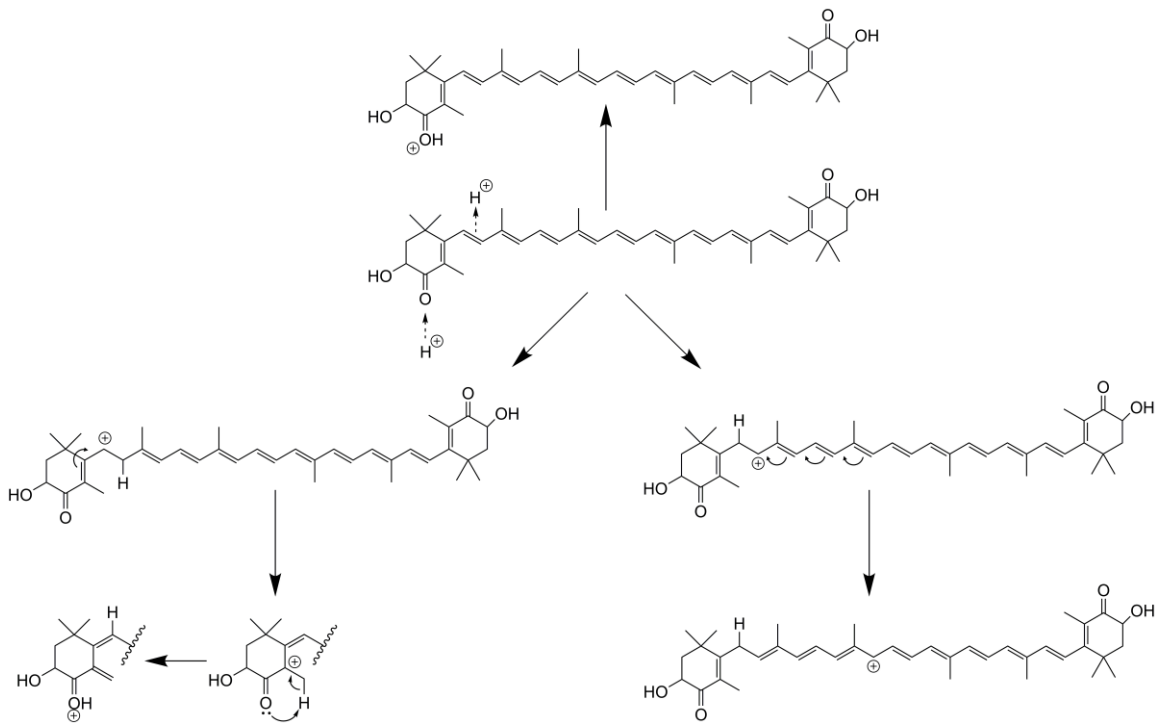
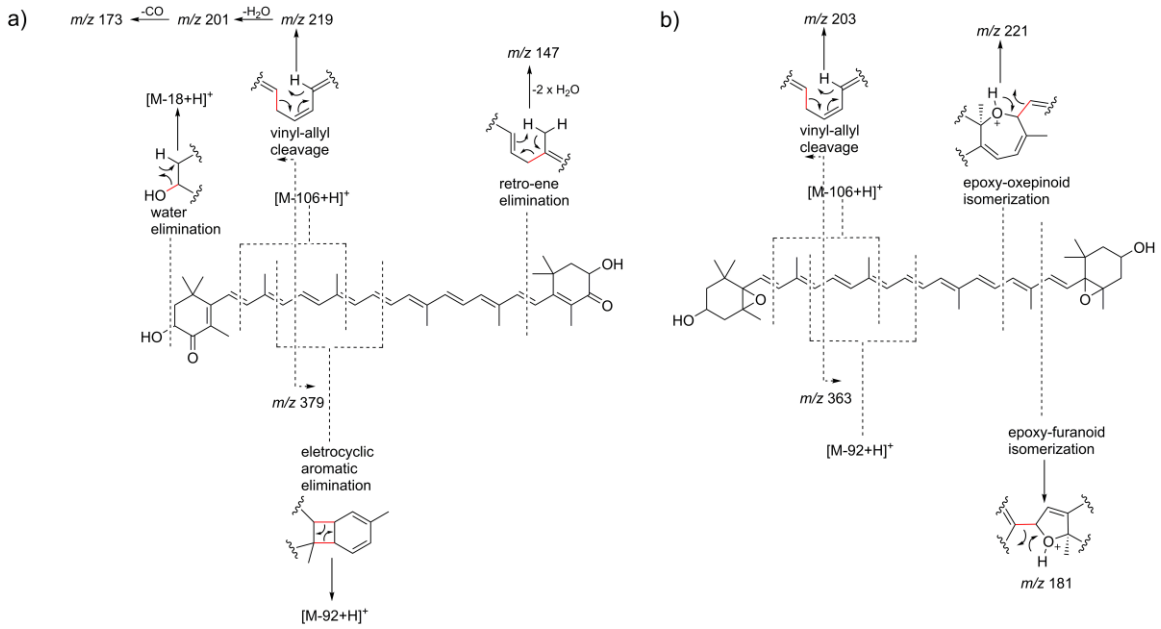


Figure 1

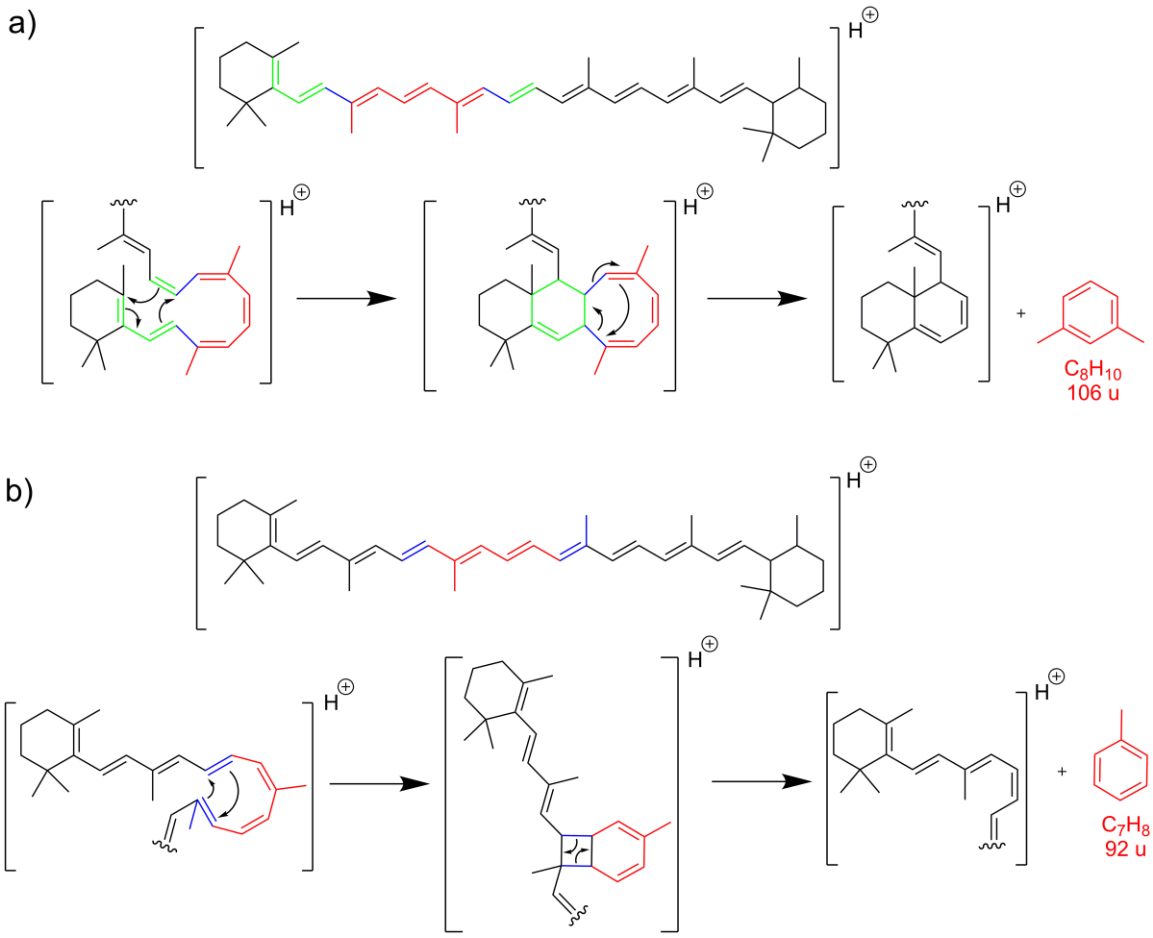


Scheme 1

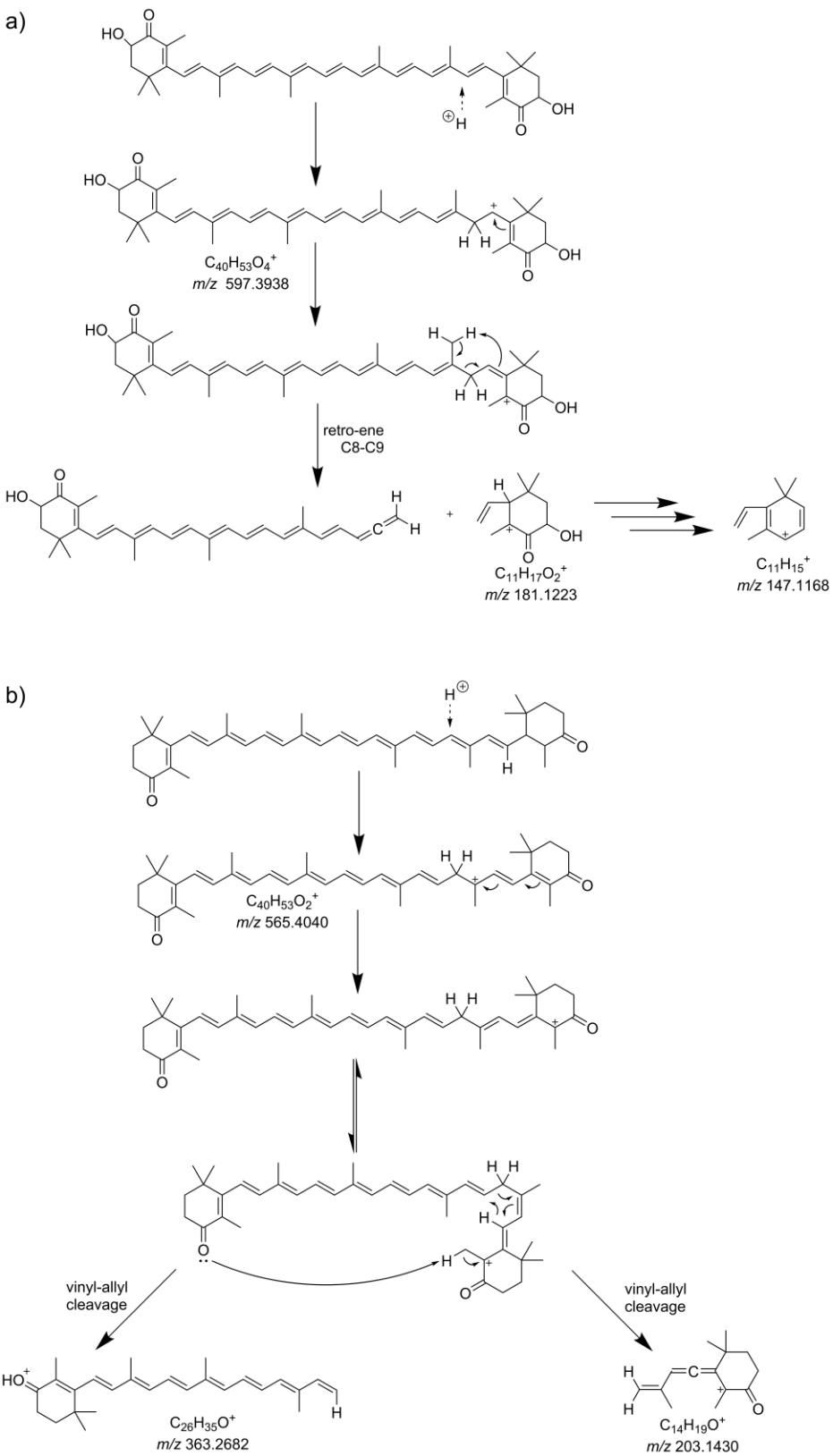


13

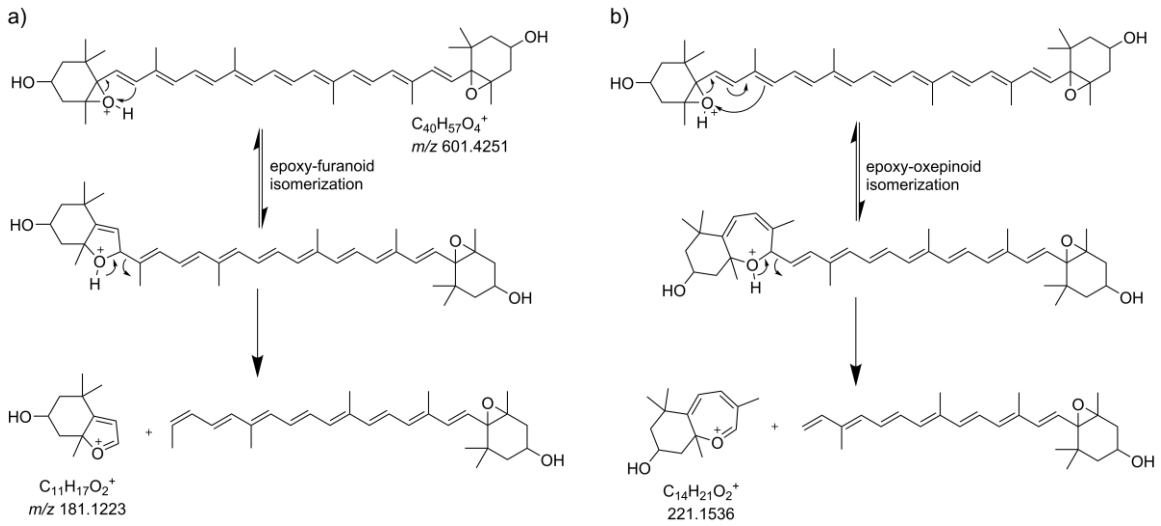
14 **Scheme** 2



Scheme 3


a)
HO
O
OH
H
$C_{40}H_{53}O_4^+$
m/z 597.3938
retro-ene
C8-C9
$C_{11}H_{17}O_2^+$
m/z 181.1223
$C_{11}H_{15}^+$
m/z 147.1168
b)
$C_{40}H_{53}O_2^+$
m/z 565.4040
vinyl-allyl
cleavage
vinyl-allyl
cleavage
$C_{26}H_{35}O^+$
m/z 363.2682
$C_{14}H_{19}O^+$
m/z 203.1430

Scheme 4


a)
b)
epoxy-furanoid isomerization
epoxy-oxepinoid isomerization
$C_{40}H_{57}O_4^+$
m/z 601.4251
$C_{11}H_{17}O_2^+$
m/z 181.1223
$C_{14}H_{21}O_2^+$
221.1536

Scheme 5

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